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# Transcriptomic analysis of colistin-susceptible and colistin-resistant isolates identifies genes associated with colistin resistance in *Acinetobacter baumannii*

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## Abstract

The emergence of colistin-resistant *Acinetobacter baumannii* is concerning, as colistin is often regarded as the last option for treating multidrug-resistant (MDR) *A. baumannii* infections. Using mRNA sequencing, we compared whole transcriptomes of colistin-susceptible and colistin-resistant *A. baumannii* strains, with the aim of identifying genes involved in colistin resistance. A clinical colistin-susceptible strain (06AC-179) and a colistin-resistant strain (07AC-052) were analysed in this study. In addition, a colistin-resistant mutant (06AC-179-R1) derived from 06AC-179 was also included in this study. High throughput mRNA sequencing was performed with an Illumina HiSeq TM 2000. In total, six genes were identified as associated with colistin resistance in *A. baumannii*. These six genes encode PmrAB two-component regulatory enzymes, PmrC (a lipid A phosphoethanolamine transferase), a glycosyltransferase, a poly-β-1,6-*N*-acetylglucosamine deacetylase, and a putative membrane protein. Matrix-assisted laser desorption/ionization time of flight mass spectrometry revealed that all three colistin-resistant strains used in this study had modified lipid A structure by addition of phosphoethanolamine. As genes found in our results are all associated with either lipopolysaccharide biosynthesis or electrostatic changes in the bacterial cell membrane, lipopolysaccharide modification might be one of the principal modes of acquisition of colistin resistance in some *A. baumannii* strains.


**Keywords:** Allelic replacement, antibiotics, lipopolysaccharide, mRNA sequencing, quantitative RT-PCR
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## Introduction

*Acinetobacter baumannii* is one of the most important opportunistic pathogens, and is responsible for diverse nosocomial infections including bacteraemia, urinary tract infections and pneumonia [1]. Polymyxins, including colistin, have traditionally not been used because of their neurotoxicity and nephrotoxicity; however, colistin is now considered the last option against multidrug-resistant *Acinetobacter* infections [2,3]. Although the current resistance rates to colistin are relatively low, they are increasing and become a serious problem worldwide [4].

In other Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Salmonella enterica*, colistin resistance is known to involve lipopolysaccharide (LPS) modification by the addition of 4-amino-L-arabinose or phosphoethanolamine. Furthermore, the PmrAB and PhoPQ two-component regulatory systems have been reported to mediate LPS modification in those species [5,6]. In *A. baumannii*, elevated expression of *pmrAB* genes related to colistin resistance has been reported [7,8]. Furthermore, mutations in the *lpxACD* genes have been shown to confer colistin resistance to *A. baumannii*, due to the loss of LPS. However, these are not the only explanations for colistin



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resistance, as colistin-resistant *A. baumannii* isolates lacking elevated *pmrAB* gene expression and lacking mutations in the *lpxACD* genes have also been identified [8]. Hence, additional mechanisms may be associated with colistin resistance in *A. baumannii*.

In this study, we used mRNA sequencing to compare whole transcriptomes of colistin-resistant and colistin-susceptible *A. baumannii* strains, with the aim of identifying genes associated with colistin resistance.

## Materials and methods

### Bacterial isolates and growth conditions and antimicrobial susceptibility testing

One colistin-susceptible (06AC-179) and two colistin-resistant (07AC-052 and 07AC-111) *A. baumannii* strains, obtained from the Samsung Medical Centre (Seoul, Korea), were used in this study. In addition, a colistin-resistant mutant (06AC-179-R1) was derived *in vitro* from a colistin-susceptible strain, 06AC-179 (Table 1) [8]. *Escherichia coli* DH5α cells were used for cloning. Selection and maintenance of transformants was achieved with 50 mg/L ampicillin and 50 mg/L gentamicin for *E. coli*, and 100 mg/L kanamycin and 50 mg/L gentamicin for *A. baumannii*. The MICs of polymyxins and other six antimicrobial agents were determined by the broth microdilution method according to the CLSI guidelines [9].

### Messenger RNA sequencing

Total RNA was extracted from two clinical strains and one mutant strain of *A. baumannii* (06AC-179, 07AC-052 and 06AC-179-R1), using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Overnight cultures were inoculated 1 : 100 in Luria–Bertani (LB) broth without colistin and grown until mid-log phase (optical density at 600 nm 0.5) at 37°C with shaking (180 rpm) for total RNA extraction. Isolated RNA was sent to Macrogen Inc. (Seoul, Korea) for further procedures. After isolation of RNA, cDNA was synthesized and sequencing libraries were generated in a strand-specific manner according to the Illumina standard protocol for high-throughput sequencing. Library sequencing was carried out with the Illumina HiSeq TM 2000 sequencer; the SolexaQA program was used to verify sequence quality. Expression levels of mRNA are expressed as reads per kilobase per million sequenced reads. The complete genome sequence of *A. baumannii* ACICU (NCBI reference sequence: NC_010611.1) was used for aligning reads.

### Quantitative real-time PCR

Genes showing a more than five-fold difference in expression level in both colistin-resistant strains and mutant strains compared with the colistin-susceptible strain were validated by quantitative real-time PCR (qRT-PCR) with standard curve method. Transcripts were quantified by qRT-PCR using SYBR Premix Ex Tag (TaKaRa, Kyoto, Japan) and a Thermal Cycler Dice Real Time System thermocycler (TaKaRa). Expression levels of targeted genes were normalized to *rpoB* expression levels. Primer sets used in this study are listed in the Supplementary material (Table S1). In *pmrAB*-deleted (Δ03002-3::Km) and its complemented mutants (Δ03002-3::Km+pJN105/03002-3), expressions of nine genes validated to be associated with colistin resistance were determined by the ΔΔCt method to compare with the expression level of 07AC-111 parental strain. Experiments were repeated with two independent cultures, each tested in duplicate.

### Allelic replacement mutagenesis

Genes validated by qRT-PCR were deleted by allelic replacement mutagenesis as previously described with some modifications [10]. We used a clinical colistin-resistant strain that was susceptible to other antibiotics, 07AC-111 (Table 1), for knockout and complementation experiments. We chose this strain because the two colistin-resistant strains used for mRNA sequencing are highly resistant to most known antibiotics; therefore, no appropriate antibiotic markers were available for selection. The linear fused products produced by the two-step PCR were cloned into pUC18, which can be used as a suicide vector in *A. baumannii*. Recombinant plasmids were introduced into the 07AC-111 strain by electroporation. Selection of

**TABLE 1. Summary for mRNA sequencing and resistance profiles of three clinical *Acinetobacter baumannii* strains and one colistin resistance-induced mutant**

| | | mRNA sequencing | | Antimicrobial susceptibility (MIC, mg/L) [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Source | Total read | Mapped read (%) | CL | PB | IMP | CPM | AMK | CIP | P/T | TIG |
| 06AC-179 | Blood | 21 214 868 | 19 117 733 (90.11) | 1 (S) | 2 (S) | >64 (R) | >64 (R) | >128 (R) | >64 (R) | >256/4 (R) | 2 (S) |
| 06AC-179-R1 | — | 15 987 058 | 14 377 187 (89.93) | >64 (R) | 64 (R) | >64 (R) | >64 (R) | >128 (R) | >64 (R) | >256/4 (R) | 2 (S) |
| 07AC-052 | Tracheal aspirate | 11 799 114 | 10 272 850 (87.06) | >64 (R) | 32 (R) | >64 (R) | >64 (R) | >128 (R) | >64 (R) | >256/4 (R) | 4 (I) |
| 07AC-111 | Tracheal aspirate | — | — | 16 (R) | 4 (R) | 0.5 (S) | 2 (S) | 4 (S) | 0.5 (S) | >1/4 (S) | 0.125 (S) |

[a]CL, colistin; PB, polymyxin B; IMP, imipenem; CPM, cefepime; AMK, amikacin; CIP, ciprofloxacin; P/T, piperacillin/tazobatam; TIG, tigecycline. R, resistant; I, intermediate; S, susceptible.

transformants was made on 50 mg/L kanamycin-containing LB plates. Transformants were confirmed by PCR and sequencing for detecting modified size of genes.

### Complementation experiments

To complement deletion mutants, the appropriate genes were amplified from genomic DNA from the 07AC-111 strain with forward and reverse primers containing *Pst*I and *Sac*I recognition sites at their 5′ ends, respectively (see Supplementary material, Table S1). The digested fragments of each gene were subcloned into the broad-host-range expression vector, pJN105, and introduced into the *E. coli* DH5α strain [11]. Recombinant pJN105 plasmids, isolated from DH5α cells, were introduced into each knockout mutant by electroporation. Transformants were selected on 0.5% arabinose-containing and 50 mg/L gentamicin-containing medium and confirmed by PCR.

### Survival analysis

The survival rates of deletion and complementation mutants were evaluated in the presence of colistin. A parental strain (07AC-111), deletion mutants and complementation mutants were each grown overnight. Bacterial cells were re-inoculated to $10^6$ CFU/mL in LB broth containing 0.5% arabinose, and incubated at 37°C for 30 min to induce the expression of genes cloned into the pJN105 vector. Cultures were grown for an additional 30 min, either with or without the presence of colistin in the medium (10 mg/L), and subsequently plated onto LB agar for colony counting. Survival rates were determined as the percentages of viable cells in LB medium containing colistin compared with viable cells in LB medium in the absence of colistin. Experiments were repeated with three independent cultures. Statistical analyses were performed with Student's *t* test using SPSS software. Differences were considered significant when $p < 0.05$.

### Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis

Lipid A was isolated from *A. baumannii* isolates by an ammonium hydroxide–isobutyric acid-based method [12]. Overnight cultures were washed twice with water and resuspended in 400 μL of isobutyric acid and 1 M ammonium hydroxide (5 : 3, vol/vol). Samples were heated at 100°C for 2 h and cooled in ice for 20 min. After centrifugation for 15 min at 2000 ***g***, supernatants were diluted with endotoxin-free water (1 : 1, vol/vol) and lyophilized. The lyophilized samples were washed twice with 1 mL of methanol. The insoluble lipid A was extracted in 200 μL of a mixture of chloroform, methanol and water (3 : 1.5 : 0.25, vol/vol). Extracted lipid A was analysed by using Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany). 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix; 2 μl of lipid A samples was mixed with an equal volume of DHB dissolved in water and acetonitrile (1 : 4, vol/vol) directly on the MALDI target and vacuum drying. Data were analysed in negative reflectron mode with a 100-Hz laser repetition rate. Three hundred shots were accumulated for each spectrum.

## Results

The three strains (06AC-179, 06AC-179-R1 and 07AC-052) used for mRNA sequencing differed only in their susceptibilities to polymyxins and tigecycline (Table 1). Illumina high-throughput mRNA sequencing generated totals of 21 214 868, 15 987 058 and 11 799 114 reads and 19 117 733 (90.11%), 14 377 187 (89.93%) and 10 272 850 (87.06%) reads were mapped from 06AC-179, 06AC-179-R1 and 07AC-052, respectively (Sequence Read Archive accession numbers, SRR1380973, SRR1449241 and SRR1408864, respectively) (Table 1). Overall, transcriptomic analysis revealed that 222 genes exhibited more than two-fold increases in expression in both colistin-resistant strains compared with the colistin-susceptible strain (see Supplementary material, Table S2).

To investigate the common genes associated with colistin resistance in both *in vitro*-selected mutant and a clinical isolate, 17 genes showing more than five-fold increased expression were selected for further studies. Validation by quantitative RT-PCR confirmed that 11 of these genes showed significantly increased expression in both colistin-resistant strains (Table 2). Overall, genes showing high fold change in mRNA sequencing were confirmed by qRT-PCR. However, ACICU_01072 and ACICU_03001 showed high fold change of over-expression in mRNA sequencing, but they were not confirmed by qRT-PCR. Although we analysed sequences in 17 genes with more than five-fold increased expression, we could not find any sequence variations between colistin-resistant and colistin-susceptible strains.

Using the allelic replacement method, nine target genes were deleted in 07AC-111. As PCR products encoding the upstream and downstream regions of ACICU_01552 and ACICU_01553 could not be obtained, possibly due to sequence polymorphisms in our strain, these genes were excluded. ACICU_03002 and ACICU_03003 are located closely adjacent to one another; hence, both genes were deleted simultaneously (07AC-111Δ03002-3).

We compared survival rates of a parental strain and the knockout mutants in the presence of colistin. Among the eight knockout mutants, all but two (Δ01518::Km and Δ02907::Km) were significantly more sensitive to colistin compared with the parental strain (Fig. 1a). Whereas 07AC-111 exhibited a

 

**TABLE 2. Genes showed different expression levels (more than five-fold) in both an induced colistin-resistant mutant (06AC-179-R1) and a clinical colistin-resistant strain (07AC-052) compared with a colistin-susceptible parent strain (06AC-179)**

| Accession no. | Description | COG [a] | Fold change (RNA-seq / qRT-PCR) | |
|---|---|---|---|---|
| | | | 06AC-179-R1 | 07AC-052 |
| **ACICU_00900** [b] | **Poly-β-1,6-*N*-acetylglucosamine (PNAG) deacetylase** | **G** | **30.54 / 59.88** | **6.17 / 6.12** |
| ACICU_01072 | Predicted membrane-associated, metal-dependent hydrolase | R | 21.83 /18.26 | 10.12 / 2.22 |
| ACICU_01158 | Putative membrane protein | — | 5.03 / 1.27 | 5.53 / 0.44 |
| **ACICU_01518** [b] | **Hypothetical protein** | — | **124.75 / 136.24** | **31.00 / 32.64** |
| **ACICU_01552** [b] | **Hypothetical protein** | — | **644.99 / 321.00** | **758.98 / 167.40** |
| **ACICU_01553** [b] | **Hypothetical protein** | — | **47.14 / 306.63** | **48.41 / 160.22** |
| ACICU_01717 | Hypothetical protein | — | 10.64 /1.24 | 18.61 / 1.10 |
| **ACICU_02866** [b] | **Glycosyltransferase** | **M** | **139.29 / 361.98** | **6.28 / 17.45** |
| **ACICU_02868** [b] | **Conserved hypothetical protein; putative membrane protein** | **M** | **96.21 / 32.75** | **6.30 / 5.42** |
| **ACICU_02895** [b] | **Non-ribosomal peptide synthetase module** | **Q** | **70.39 / 162.30** | **11.20 / 32.00** |
| **ACICU_02907** [b] | **Diacylglycerol kinase** | **M** | **46.26 / 23.32** | **14.52 / 5.60** |
| ACICU_03001 | PAP2 (acid phosphatase) superfamily protein | R | 52.75 /1.41 | 9.26 / 0.46 |
| **ACICU_03002** [b] | **Signal transduction histidine kinase (PmrB)** | **T** | **20.21 / 18.59** | **5.51 / 5.57** |
| **ACICU_03003** [b] | **Response regulator consisting of a CheY-like receiver domain and a Winged-helix DNA-binding domain (PmrA)** | **T, K** | **24.95 / 30.06** | **6.27 / 6.66** |
| **ACICU_03004** [b] | **Lipid A phosphoethanolamine transferase (PmrC)** | **R** | **78.65 / 13.43** | **14.89 / 432.2** |
| ACICU_03426 | Acetyl-CoA hydrolase | C | 6.33 /1.02 | 5.13 / 1.17 |
| ACICU_03497 | Predicted membrane protein | S | 13.05 /1.75 | 5.94 / 1.09 |

[a]C, Energy production and conversion; G, Carbohydrate transport and metabolism; K, Transcription; M, Cell wall/membrane/envelope biogenesis; Q, Secondary metabolites biosynthesis, transport and catabolism; R, General function prediction only; S, Function unknown; T, Signal transduction mechanisms.
[b]Genes confirmed with quantitative RT-PCR are indicated in bold type.

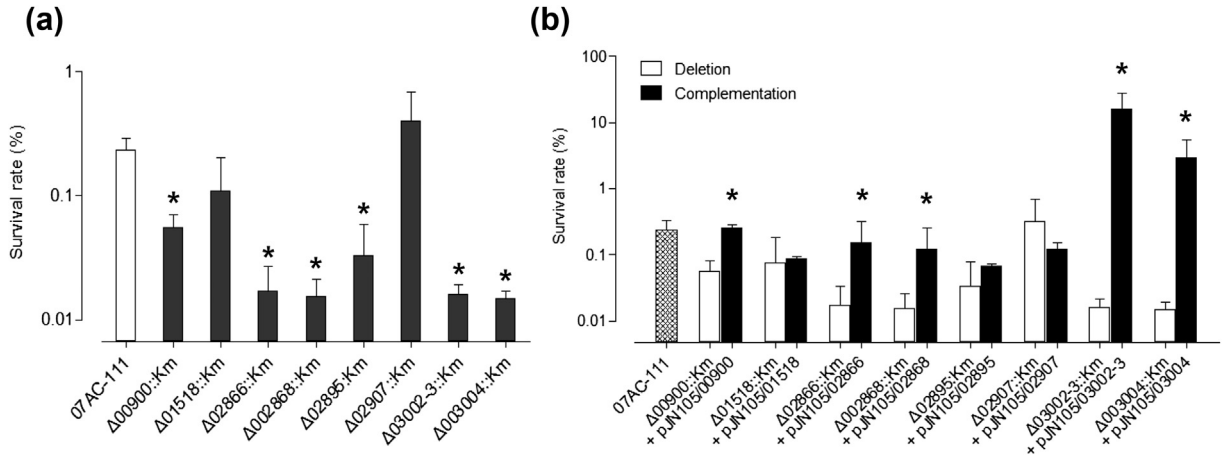


**FIG. 1.** Survival rates in the presence of colistin of different *Acinetobacter baumannii* strains. Survival rates in the presence of colistin (10 mg/L) were determined for (a) the 07AC-111 parental strain and eight knockout mutants, and (b) six of the knockout mutants showing significantly decreased survival rates, and their complemented equivalents. All experiments were performed in triplicate. *$p < 0.05$. The dotted line indicates the survival rate of the parental strain, 07AC-111. Error bars represent the standard deviations of three biological repeats.

survival rate of 0.18% in the presence of 10 mg/L colistin, the survival rates of six deletion mutants ranged from 0.013% (Δ03004::Km) to 0.07% (Δ00900::Km).

Six knockout mutants, each showing increased sensitivity to colistin, were complemented with pJN105 containing the appropriate gene. Survival rates in the presence of 10 mg/L colistin were restored in all complemented strains but one, 07AC-111Δ02895::Km+pJN105/02895 (Fig. 1b). In particular, the survival rates of Δ03002-3::Km+pJN105/03002-3 and Δ03004::Km+pJN105/03004 increased >1576-fold and >325-fold, respectively, compared with the parental strain. This finding underscores the importance of *pmrCAB* genes in colistin resistance in *A. baumannii*. In a *pmrAB*-deleted mutant (Δ03002-3::Km), the expression of ACICU_00900, ACICU_01518, ACICU_01552, ACICU_02866, and ACICU_03004 decreased significantly. In addition, their expression was restored in its complemented mutant (Fig. 2). Although ACICU_01553 and ACICU_02868 were over-expressed in parental isolate and complemented mutant, the results were insignificant or inconsistent. On the other hand, ACICU_02895 and ACICU_02907 may not be associated with the deletion or complementation of *pmrAB*.

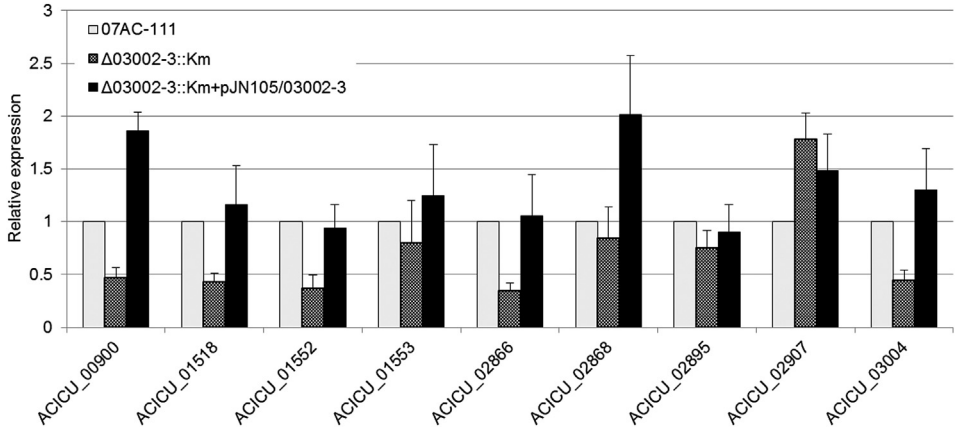


**FIG. 2.** Gene expressions in *pmrAB*-knockout and -complemented mutants by quantitative RT-PCR. Relative expressions of nine genes were determined using ΔΔCt method. Error bars represent the standard deviations of three biological repeats.

Antimicrobial susceptibility of knockout and complemented mutants was evaluated. As a result, MICs of knockout and complemented mutants were not changed for all antibiotics tested, compared with wild-type strain 07AC-111 (Table 1).

In MALDI-TOF MS, two common peaks found in all four isolates correspond to a hepta-acylated lipid A with two 2-amino-2-deoxyglucose residues, two phosphates, three 12:0(3-OH), two 14:0(3-OH) and two 12:0 (*m*/*z* 1,910) and to a tetra-acyl lipid A with three 12:0(3-OH) fatty acids and one 14:0(3-OH) fatty acid (*m*/*z* 1,404) attached to the phosphorylated disaccharide (Fig. 3). Three colistin-resistant isolates had a peak at *m*/*z* 2034, which indicates an addition of phosphoethanolamine (*m*/*z* 124) to the normal hepta-acylated structure. An induced colistin-resistant strain 06AC-179-R1 showed additional two peaks at *m*/*z* 1937 and *m*/*z* 1954 compared with susceptible parental strain. The peak at *m*/*z* 1,937 might be bis-phosphorylated hexa-acylated lipid A with the addition of one residue of phosphoethanolamine and one residue of 2-carbon ethanolamine (*m*/*z* 85) [13]. It is considered that the peak at *m*/*z* 1954 might be bis-phosphorylated hepta-acylated lipid A with the removal of one phosphate group (*m*/*z* 80) and the addition of one residue of phosphoethanolamine [14].

## Discussion

In this study, we aimed to identify candidate genes responsible for colistin resistance by comparing whole transcriptomes from colistin-susceptible and colistin-resistant strains. Of these 17 candidate genes exhibiting more than five-fold increased expression in both the clinical and *in vitro*-derived colistin-resistant strains, 11 were validated by qRT-PCR. Through survival analysis in the presence of colistin of deletion and complementation mutants, this study identified a role for six

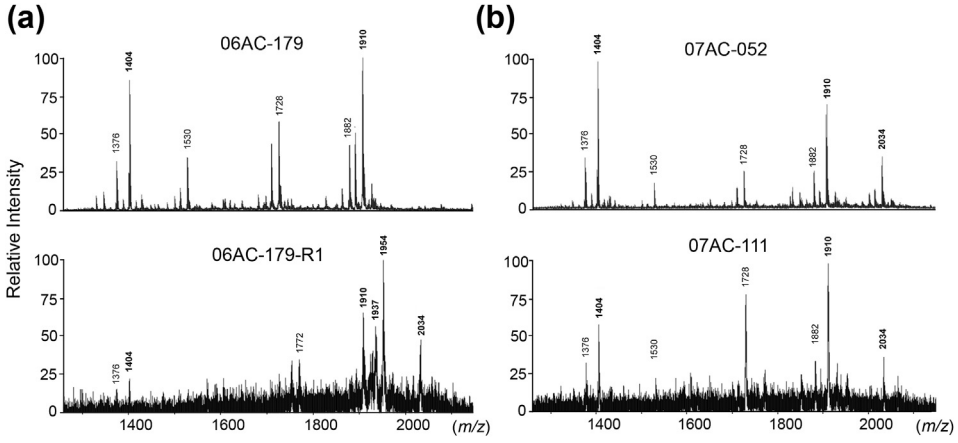


**FIG. 3.** Mass spectrometry of lipid A extracted from four *Acinetobacter baumannii* strains used in this study. (a) The colistin-susceptible parental strain 06AC-179 and induced resistant mutant 06AC-179-R1. (b) Clinical colistin-resistant strains 07AC-052 and 07AC-111.

genes (ACICU_00900, ACICU_02866, ACICU_02868, ACICU_03002, ACICU_03003 and ACICU_03004) in colistin resistance in *A. baumannii*.

The involvement of ACICU_03002 to 03004 in colistin resistance is not surprising as they are homologues of *pmrBAC*, respectively [7]. Since *pmrC* is known to function as a phosphoethanolamine transferase, we determined structures of lipid A from all isolates used in this study by MALDI-TOF MS. As expected, all three colistin-resistant isolates including induced resistant isolate 06AC-179-R1 were found to have the modified lipid A by addition of phosphoethanolamine.

This study also indicates that upregulation of ACICU_02866 encoding a glycosyltransferase contributes to the colistin resistance in *A. baumannii*. Glycosyltransferases involved in the synthesis of LPS cores were shown to be significant in colistin resistance in *A. baumannii* [15,16]. Despite the increased glycosyltransferase activity, an additional lipid A modification of LPS was not found by MALDI-TOF MS. ACICU_02868, which consists of the same operon as ACICU_02866, was upregulated in both the colistin-resistant isolate and the induced resistant isolate. The deletion and complementation experiments also revealed that ACICU_02868 may be associated with colistin resistance. It is suggested that upregulation of the operon ACICU_02865 to ACICU_02868 may be associated with colistin resistance or adaptation to colistin in *A. baumannii*.

Regarding the association between cell membrane integrity and colistin resistance, overexpression of ACICU_02907 encoding a diacylglycerol kinase (DAGK) is observed in colistin-resistant strains. In *E. coli*, DAGK is a small integral membrane protein [17], whose function is to recycle the population of diacylglycerol that is generated largely as a by-product of membrane-derived oligosaccharide biosynthesis [18]. Hence, overproduction of DAGK may increase the integrity of the cell membrane, so aiding colistin resistance, although more investigations would be required.

It was shown that ACICU_00900 (or *pgaB*), a gene encoding a poly-β-1,6-*N*-acetylglucosamine (PNAG) deacetylase, may be associated with colistin resistance in *A. baumannii*. PgaB has been shown to be associated with transport of the surface polysaccharide, PNAG [19]. In *E. coli*, changes in PNAG production have been shown to influence biosynthesis of the LPS core [20]. In addition, upregulation of PNAG transport and increased production of PNAG were identified in a transcriptomic study of an LPS-deficient (i.e. colistin-resistant) *A. baumannii* strain [15]. Although neither of our colistin-resistant strains showed abnormal expression of any of the genes related to LPS biosynthesis, overexpression of positively charged PNAG may inhibit the action of cationic colistin on LPS. In addition, polysaccharide deacetylase is involved with cell wall modification [21]. Much higher increased expression of ACICU_00900 in the complemented mutants than in the knockout mutant of *pmrAB* suggests that the production of PNAG may be regulated by the PmrAB regulatory system (Fig. 2), although further investigation is needed. In a recent study, the extent of *A. baumannii* cell wall damage was shown to correlate with the concentration of colistin treatment; furthermore, cell wall damage was delayed and less severe in colistin-resistant strains [22]. Hence, cell wall modifications may influence the uptake of antibiotics, including colistin, so resulting in antibiotic susceptibility. Hence, a glycosyltransferase, PNAG deacetylase, and other unidentified enzymes may constitute an interaction network that influences the integrity of the outer membrane and cell wall, resulting in the colistin resistance in *A. baumannii*.

In other bacterial species such as *S. enterica*, *Yersinia pestis* and *Klebsiella pneumoniae*, the PmrAB system, which mediates expression of the *pbgP* operon, is regulated in concert with the PhoPQ system, in a feed-forward loop manner [23]. As the PhoPQ system is absent in *A. baumannii*, the PmrAB system may respond alone to environmental stimuli and promote colistin resistance. Otherwise, there may be a cross-talk between the PmrAB system and other unknown regulators, and a different system may be involved in colistin resistance in *A. baumannii*. In addition, we found that complemented Δ03004::Km+pJN105/03004 had a survival rate much higher than the knockout mutant. It confirms that the addition of phosphoethanolamine to lipid A may give a great contribution in colistin resistance in *A. baumannii*, because the phosphoethanolamine was directly transferred, not mediated by the PmrAB regulatory system [24]. In this study, although some genes associated with colistin resistance including ACICU_00900, ACICU_01518, ACICU_01552, ACICU_02866 and ACICU_03004 may be under the transcriptional network of PmrAB system, other genes such as ACICU_02895 and ACICU_02907 may not be related to the PmrAB system.

In this study, we identified and validated six genes encode PmrAB two-component regulatory enzymes, PmrC, a glycosyltransferase, a PNAG deacetylase, and a putative membrane protein by transcriptomic analysis. All of these gene products are thought to be associated with either LPS biosynthesis or modification of its electrostatic properties, thereby confirming that modification of LPS is one of the principal methods for acquiring colistin resistance. However, our cut-off for selecting follow-up candidates may be too stringent to identify all the genes associated with colistin resistance; hence, additional colistin resistance mechanisms may also exist. Importantly, our study also shows that comparative transcriptomic analysis is a useful approach for investigating antibiotic resistance mechanisms.

## Transparency declaration

The authors declare no conflicts of interest.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.04.009.

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